

The University of Chicago

DIRECT BACTERIOLOGICAL EXPERI-
MENTATION ON THE LIVING
MAMMALIAN FETUS

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

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By
ORAM CLARK WOOLPERT

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DIRECT BACTERIOLOGICAL EXPERIMENTATION ON THE LIVING MAMMALIAN FETUS *

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INTRODUCTION

The concept of parasitism as a general biological problem cannot be complete without a consideration of the reactions of embryonic and fetal animals. The newborn is not a *tabula rasa* upon which the principles of immunology may be inscribed. It is relatively mature in form and function and reflects in large measure the resistance or susceptibility characteristic of its species. We do not know whether these characteristics are manifest in the youngest embryos or become gradually apparent during the intricate growth period from inception to birth. If susceptibility in any degree parallels morphological development, we may expect fetal reactions to differ widely from those of postnatal animals.

Fragmentary information on the occurrence of spontaneous infections of the fetus has accumulated and congenital transmission of many of the infectious diseases is known to be possible. Such transmission is only occasional and usually near term. More data are necessary before we can judge whether the younger fetuses are more resistant to infection or merely less exposed thereto.

Most of the investigations dealing with experimental fetal infections have been concerned with passage of the infectious agent from maternal to fetal circulations through the placenta. In experiments of this type the amount of inoculum that actually reaches the fetus and the time of effective fetal inoculation remain unknown. Many factors affect the result, such as the concentration of the organism in

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the maternal blood stream, the species type of placenta, and the degree of placental damage.

In view of these considerations a more direct approach to the problem of parasitism of the fetus appears desirable. But further than this, there is ample justification on a practical basis for this type of experimentation. Much of the work of the bacteriologist is dependent on the use of experimental animals in the study of both bacteria and viruses. Yet we know that there are definite and characteristic limitations in the use of any particular animal for any given parasite. It is important to determine whether some of these limitations might be modified by extending the host range to include the fetus. Furthermore, the fetus occupies a unique situation, being in an environment of constant temperature and nutrition and well protected from chance contamination and external influence. It constitutes what might be termed a "test tube animal." Such conditions appear ideal for the facilitation of certain types of bacteriological studies.

TECHNIQUE

Isolated reports of fetal experimentation may be found in the literature published by Charrin,¹ Kreidl and Mandl,² Wolff,³ Bourquin,⁴ Bors,⁵ Nicholas,⁶ Debrunner,⁷ Wohlwill and Bock⁸ (1933), and others. These studies have been concerned chiefly with pathological changes or with the passage of various substances through the placenta. The work of Bors particularly established the practicability of experimentation on mammalian fetuses.

It is apparent that the purposes of the bacteriologist will not usually require such an elaborate technique as might be demanded for delicate intrauterine surgery in anatomical or similar studies. In the work reported herein we have used the most simple technique consistent with well controlled bacteriological procedure.

Guinea pigs, rabbits and a few rats were used. Most of the work was done on the guinea pig and the remarks herein will refer to that animal unless otherwise indicated. In general, we found the guinea pig the most adaptable to this type of experimentation. They care for their young better, have a longer period of gestation, and tolerate repeated surgical procedures well. The stock animals were kept in large breeding pens and suitable pregnant animals selected from time to time. With a little experience one can judge the approxi-

mate fetal age by palpation. This is possible from about the 20th day of gestation until term at about the 65th day.

Several different methods of inoculation were used, depending upon the fetal age. Until about the 30th day the fetus is so small in relation to decidua, placenta and amniotic fluid that direct inoculation is impracticable. For this stage intraplacental inoculation was employed. The mothers were anesthetized either by drop ether from a separatory funnel, by sodium amytal (Lilly) given hypodermically in a dosage of about 50 mg. per kilo body weight, or by a smaller dose of sodium amytal followed by ether. Following mid-line laparotomy under aseptic precautions, the uterus was delivered, the number and location of fetuses in the two uterine horns determined, and the placentas inoculated by needle puncture through the uterine wall. The abdominal wall was closed in one layer, the skin with heavy interrupted sutures, and collodion was applied to the incision. The placental method of inoculation is necessarily open to the objection that there can be no control of the distribution of inoculum between maternal and fetal blood sinuses in the placenta.

Fetuses from about 30 to 40 days of age were inoculated by needle puncture through the maternal uterine wall and fetal membranes, following surgical exposure of the uterus as described above. At this age the fetuses are readily palpable, the fetal head particularly being easily identified and inoculated. Most of our inoculations were made at this period of gestation and in this way. Needle puncture through the uterine wall is associated with a certain amount of contamination of maternal tissue with inoculum intended for the fetus. In most of our experiments such maternal contamination was not considered important. To avoid this, in certain instances the fetus was exposed by a small incision through the uterine wall and fetal membranes. Following direct needle inoculation the site was cauterized to destroy surface traces of the inoculum.

Older fetuses are more likely to be aborted if the mother is subjected to the full operative procedure. In some cases such fetuses were inoculated through small incisions in the maternal abdominal wall immediately over the fetal part desired. We found it possible also to inoculate older fetuses intracerebrally by needle puncture through the intact maternal abdominal wall. The fetal calvarium becomes calcified long before term and in the later fetal stages can be identified and manipulated against the maternal abdominal wall,

particularly if the mother is well relaxed with anesthesia. Although the danger of perforating some interposed viscus appears great, in no instance was there any evidence that such a thing occurred, nor did any of these mothers abort.

The termination of our experiments was principally by cesarean section. With such a procedure the several fetuses could be identified by their respective positions in the uterus. If the mother is allowed to go to term such identification is not possible and there is the danger that some of the fetuses born dead or alive may be destroyed by the mother. In several instances we have performed serial cesarean sections a week or more apart on the same litter. Occasionally we have delivered mature fetuses by section in order to identify them and then permitted them to be reared by foster mothers. Usually the mothers were sacrificed at section but in many cases the operations were completed surgically, the mothers were re-bred and used again, some of them repeatedly, in similar experimentation.

EXPERIMENTAL

The following antigens were selected for inoculation in fetal guinea pigs:

- (1) The virus of poliomyelitis, for which the monkey is at present the only susceptible experimental animal.
- (2) The vaccinia virus, which finds a relatively resistant host in the guinea pig.
- (3) The submaxillary gland virus of guinea pigs, a virus natural to that animal and latent in spontaneous infections.
- (4) The tubercle bacillus (H37) and Bacille Calmette-Guérin (B C G), representing virulent and relatively non-virulent strains, respectively.
- (5) Diphtheria toxin, for the study of which the guinea pig may be said to be the classical experimental animal.

(1) *Poliomyelitis Virus*

A chemically purified and concentrated virus of known infectivity for monkeys was passed through two series of fetal guinea pigs, one at 5 day, the other at 10 day intervals. Eight transfers were made in the former series and four in the latter. The fetuses were 30 to 50 days old. All inoculations were made intracerebrally by needle

puncture through the uterine wall. Fetuses to be used as a source of subinoculation material were removed through cesarean section. From each fetus of the litter one lateral half of the brain was removed aseptically for subinoculation; the other half was preserved for histological examination. The passage material was prepared by pooling the brain tissue from the several fetuses of the litter, grinding with sand in a sterile test tube and suspending in saline solution.

In none of the fetuses were there gross or histological changes that could be interpreted as representing a specific effect of the virus. Material from the sixth subinoculation (5 day series) failed to produce poliomyelitis administered intracerebrally to a monkey. Frequent bacteriological cultures of fetal brain and heart blood in beef heart broth and on blood agar plates were consistently negative.

Comment: The above experiments are of course not extensive enough to warrant final conclusions as to the susceptibility of this fetal animal to poliomyelitis virus. Inoculations by other routes, at other ages, through larger series, and in other species would be necessary for complete exploration of the problem. From work reported by Stritar and Hudson⁹ on the vaccinia virus, it is evident that the concentration of a virus can vary greatly in the different fetal organs. Perhaps the poliomyelitis virus would find a more favorable medium for growth in some fetal organ other than the brain. Within the limitations of the experiment, however, it is clear that the fetal guinea pig manifested the resistance characteristic of its species to poliomyelitis virus infection. The fact that the fetal tissues remained bacteriologically sterile throughout the course of transfer is noteworthy in view of claims of a bacterial form of the virus.

(2) *Vaccinia Virus*

A neurotropic strain (Levaditi), maintained in tissue culture, was employed. Its dermal titer in rabbits was 1:10,000. When injected intracutaneously in adult guinea pigs in 1:10 dilution it caused only a faint local reaction and no febrile response. Administered intracerebrally to fetal guinea pigs in 1:100 dilution it was found to disseminate widely and often produced death within 5 to 7 days. The sites of predilection for gross lesions seemed to be skin and lungs, although lesions were occasionally seen in liver, brain and elsewhere. The cutaneous lesions stood out sharply on the hairless fetal skin as

pale, slightly raised nodules from 0.5 to 3 mm. in diameter, some of which progressed to ulceration. The virus could be recovered in high titer from these lesions and its specificity demonstrated by rabbit inoculation.

Comment: This is an example of a fetal animal that appears to be much more susceptible than the newborn or adult to a particular virus infection. Although guinea pigs have at times been used extensively in ophthalmic tests for vaccinia virus, and Bland¹⁰ has shown that the virus may be adapted to the guinea pig by serial transfer to the degree that it causes skin lesions, there is a general and well founded belief that the guinea pig is relatively resistant to vaccinia infection.

(3) *Submaxillary Gland Virus of Guinea Pigs*

Four different preparations of fresh guinea pig submaxillary gland material were injected into fetal and newborn guinea pigs concurrently.* The results may be summarized by stating that for all four preparations there was a positive correlation between the effects in fetal and newborn animals. One of the preparations was innocuous in both. The other three were effective in dilutions up to 1:1000 in the fetus. Widespread and characteristic lesions and sometimes death resulted. The effects on individual fetuses of a litter were proportionate to the amount of the material injected and the duration of the experiment.

Comment: In this instance we are exposing the fetal guinea pig to a virus native to the species yet usually latent in spontaneous infections. Most adult guinea pigs may be considered to possess an "infection immunity," since they manifest no active symptoms of the disease and still carry the virus in an infectious state. Interesting problems in immunology are thus raised. One might expect that immune adults would passively protect their young. However, Markham and Hudson¹¹ have observed that fetuses can be definitely infected even though borne by spontaneously infected mothers. One may infer, therefore, either that the maternal immunity is not humoral or that antibodies do not pass the placenta in effective quantity.

* This material was obtained from Dr. F. S. Markham and the inoculations of newborn guinea pigs referred to were made by him (Markham and Hudson¹¹).

In these experiments and others the practicability of titration of infectious material in a single litter has been seen. In no case has there been any evidence of transfer of the infectious agent from one fetus to another within the same litter. Control fetuses have never been involved.

(4) *Bacillus Tuberculosis* and *Bacille Calmette-Guérin*

The H₃₇ virulent human strain of *B. tuberculosis*, grown on Long's synthetic medium, was prepared in weighed amounts for inoculation as a suspension in saline. The guinea pig fetuses were injected intracerebrally, for the most part. All such fetuses died within a short time, some *in utero* and others soon after birth. Characteristic changes, both local and metastatic, were evident. Meningitis and necrosis of the fetal brains were often very marked. The liver and spleen usually contained tubercles if the infection had persisted for more than a few days.

The B C G was prepared and injected in like manner. Again, changes characteristic of tuberculosis resulted. Tendency to distribution, however, was much less, and the local lesions were less severe than those produced by comparable dosages of the virulent tubercle bacillus.

Comment: Since the guinea pig is a classical animal for tuberculosis work, the susceptibility of the fetal guinea pig is not surprising. Whether the fetus is relatively more susceptible than newborn or adult animals was not determined in these preliminary experiments. They are presented here to illustrate possible advantages of using the fetus in virulence tests. Such experiments have always been open to the objection that the animal may become accidentally infected with extraneous organisms. The fetus, however, is not subject to chance infection, so that the effects of direct inoculation or of serial passage must be given full credence (Neiman and Woolpert¹²).

A point of interest in fetal experiments on tuberculosis was that pathological changes often proceeded *in utero* far beyond the stage that would have been compatible with life in the external environment. The brains of some of these fetuses were almost entirely destroyed, yet the animals lived and developed normally, succumbing promptly, however, when delivered into the outer world as autonomous beings.

(5) *Diphtheria Toxin*

A ripened, standardized toxin, obtained through the courtesy of Parke, Davis & Co., was titrated in 6 newborn guinea pigs and 8 fetuses in three litters. The actual amounts of toxin injected into the fetuses ranged from 0.1 cc. of 1:1280 dilution to 0.1 cc. of 1:20 dilution. All of the fetuses were delivered by section from 2 to 9 days following inoculation. Protocols for the fetal inoculations are set forth in Table I. Table II gives a summary of the results

TABLE I
Diphtheria Toxin Titration in Fetal Guinea Pigs

Guinea pig No.	Approximate days of pregnancy	Day experiment terminated	Fetus No.	Approximate weight of fetus	Inoculum		Results
					cc.	Dilution of toxin	
291	40	49	1	gm. 10	0.10	1:1280	Living; weight 31.2 gm., grossly normal
			2	10	0.10	1:640	Living; weight 31.6 gm., grossly normal
290	35	37	1	5	0.05	1:320	Living; weight 5.5 gm., grossly normal
			2	5	0.05	1:160	Living; weight 6.8 gm., grossly normal
			3	5	0.05	1:80	Dead; weight 6 gm., marked postmortem change
295	38	40	1	10	0.10	1:80	Dead; weight 10 gm., early postmortem change
			2	10	0.10	1:40	Dead; weight 12.5 gm., early postmortem change
			3	10	0.10	1:20	Dead; weight 10.3 gm., extensive postmortem change

computed on a basis of cubic centimeters of toxin per kilogram body-weight of animal and shows that the minimal lethal dose for both newborn and fetus was between 0.05 and 0.10 cc.

Comment: These experiments imply that the fetal guinea pig is as reactive as the newborn to the action of this toxin; in fact there is a surprising agreement of titer, considering the small number of animals used. It was shown by Schmidlechner¹³ in 1904 that fetal guinea pigs could be killed *in utero* if sufficient toxin were injected into the mother to provide an excess over the amount that would be bound by the maternal tissue. Nevertheless, the impression has

been general that young animals are relatively insusceptible to classical toxins because of a lack of "receptors" or for other theoretical reasons. The fetuses used in our experiments were from one-twentieth to one-fifth the weight of newborn guinea pigs, yet they were acutely sensitive to the small amounts of diphtheria toxin injected. Histological studies were not made. Again, the practicability of titrations in fetal litters is suggested.

TABLE II

Comparative Reaction of Newborn and Fetal Guinea Pigs to Diphtheria Toxin

Newborn guinea pigs		Fetal guinea pigs	
0.1	cc. per kilo — lethal	0.5	cc. per kilo — lethal
0.05	" " " — sublethal	0.25	" " " — lethal
0.033	" " " — sublethal	0.125	" " " — lethal
		0.0625	" " " — sublethal
		0.0312	" " " — sublethal
		0.0156	" " " — sublethal
		0.0073	" " " — sublethal

DISCUSSION

Our interest in the fetus as an experimental animal was the outgrowth of efforts to find a more convenient animal than the monkey for poliomyelitis studies. Although this hope failed of realization there appeared sufficient promise in the work to warrant extending its scope to include other infectious agents. The major technical problems encountered were: first, that of inoculating the fetuses without jeopardizing the life of the mother, or producing abortion; and second, that of observing the course of events following inoculation and recovering the virus and fetal tissues at the stage desired. In our experience maternal death occurred rarely and abortion did not commonly take place if healthy mothers at the appropriate gestation stage were used. Graded inoculations and a few trial experiments with the particular virus enabled us to judge fairly accurately the proper time for terminating an experiment. It is hoped that the technique can be modified to permit experimentation on both younger and older fetuses and to include other species. The actual experimental results in this report should be considered as preliminary and suggestive rather than final. More extensive and detailed

experiments are reported elsewhere by Neiman and Woolpert,¹² Stritar and Hudson,⁹ and Markham and Hudson.¹¹

Any generalizations in theory concerning fetal resistance to infection would be unwarranted at this time. *A priori*, one might favor one or the other of two views: either the fetus in its primitive state should constitute merely good culture medium, devoid of cellular or humoral defense; or, on the other hand, it should be resistant because of its very immaturity. So far as histological reaction is concerned, the impression is general that embryos and younger fetuses are relatively non-reactive. Wohlwill and Bock⁸ (1930) could not demonstrate inflammatory reaction in the human fetus under 11.5 cm. in length. From this stage on, a localized histiocytic reaction became gradually evident. Granulocytic infiltration was seen only in the more mature fetuses. In experimental studies the same authors⁸ (1933) found that fetal guinea pigs were histologically relatively non-reactive to the bacterial and chemical irritants used. However, lack of histological response does not necessarily imply absence of tissue damage or unsuitability for the support of bacterial growth. We know that embryonic tissues have been found particularly favorable for the propagation of many viruses in tissue culture. In our experience with the fetal guinea pig there were marked tissue changes and often death associated with infection by the tubercle bacillus and the submaxillary gland virus of guinea pigs. The histological changes produced by vaccinia virus were less extensive but death often resulted. Although fetuses were killed by small amounts of diphtheria toxin there was probably little characteristic gross change. The poliomyelitis virus exerted neither vital nor cellular effects. It may be that fetal reactions, like those of adults, will be found to be individualized, with respect to both host and parasite.

SUMMARY

A technique for direct bacteriological experimentation on the living fetus of small laboratory mammals is described.

The theoretical and practical advantages of such experimentation are discussed.

The results of preliminary experiments illustrating the applications and limitations of this technique are briefly reported.

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